European Review for Medical and Pharmacological Sciences 2020; 24: 10687-10695

MiR-340 suppresses CCl4-induced acute liver injury through exerting anti-inflammation targeting Sigirr

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Abstract. – OBJECTIVE: Acute liver injury (ALI) can be caused by ischemia, viral infection, immune disorders and exogenous substances. Finding novel drugs and methods to treat liver injury is still one of the problems to be precipitously solved in clinical liver diseases. Recent studies have verified that microRNA-340 (miR-340) and the Single immunoglobin interleukin-1 (IL-1)-related receptor (Sigirr) display extensive anti-inflammatory effects against inflammatory diseases. However, their protection against inflammation in ALI is unclear. The purpose of this study was to investigate the regulated mechanism of miR-340 targeting Sigirr on ALI.

MATERIALS AND METHODS: Firstly, the expressions of miR-340 and Sigirr in different time of inflammatory Kupffer cells (KCs) were detected. Lipopolysaccharide (LPS) was employed in activating the KCs inflammation, and tetrachloromethane (CCl4) was performed to induce liver injury. Then miR-340 mimic and inhibitor were used to up-regulate or down-regulate the function of miR-340 to explore anti-inflammation function to ALI *via* the target of Sigirr.

RESULTS: The study results exhibited that the expressions of miR-340 and Sigirr were markedly decreased in LPS-induced KCs inflammation, and CCl4 induced the development of ALI. Besides, the overexpression of miR-340 could alleviate the inflammation of LPS induction in KCs *via* promoting Sigirr. Moreover, miR-340 and Sigirr rescue significantly reduced liver function and tissue lesion by employing miR-340 mimic.

CONCLUSIONS: MiR-340 decreases KC inflammation *via* enhancing Sigirr, but accumulating miR-340 prevents inflammation damage and ameliorates ALI. In addition, increased miR-340 and Sigirr may become novel targets for the therapy of ALI in the future.

Key Words:
MiR-340, Kupffer cells, Acute liver injury, Sigirr, Anti-inflammartion.

Introduction

Acute liver injury (ALI) is a sudden abnormal hepatic dysfunction caused by a variety of factors in a short period of time[1]. Drug poisoning, virus infection, immune response, and ischemia-reperfusion are the most common inducing factors of ALI[2-4]. ALI can induce a series of complications and also aggravate the progress of other diseases, which should be actively prevented, and treated. Most ALI patients can recover with medication, but some patients will progress to acute liver failure with high mortality[5]. Inflammatory response-mediated ALI is caused by the massive release of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL), resulting in activation of the nuclear factor NF-κB pathway[6,7]. Activation of NF-κB pathway leads to the expressions of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which eventually results in the synthesis of a large number of inflammatory mediators, such as prostaglandins (PGs) and NO, and mediates the occurrence of inflammatory response[8,9]. MiRNAs (microRNAs), first discovered by Ambros et al[10] in 1993, can exist in a variety of body fluids but mainly distributed in serum and plasma[11,12]. They are a kind of non-coding RNAs with a length of about 18-24 nucleotides, which inhibit the translation of target gene mRNAs or promote mRNA degradation by partially or completely complementing the 3′-untranslated region (3′-UTR) of target mRNAs, so as to achieve post-transcriptional regulation of the expression level of protein-coding gene[13,14]. MiR-340, as a miRNA with anti-inflammatory effects, plays a potential therapeutic role in a variety of diseases, including neuroinflammation[15], cancer[16], and

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multiple sclerosis[17]. Sigirr, a member of the TIR superfamily, has been shown in recent studies to negatively regulate the innate immune response mediated by IL-1R/TLRS receptor, and plays an important role in the regulation of inflammatory response induced by infectious diseases, tumors, and autoimmune diseases[18]. However, there is no reported explanation of the molecular regulation mechanism of Sigirr in ALI. In this study, miR-340 was found to target the regulation of Sigirr to play an anti-inflammatory role in KCs. Besides, the overexpression of miR-340 could significantly increase Sigirr level to inhibit the inflammatory response caused by over-activation of KCs after ALI and ameliorate liver tissue damage and decrease of liver function.

Materials and Methods

Human KC Culture and Transfection

Human KCs were obtained from People's Hospital of Zoucheng and were cultured in 5×5 cm_2 cell flasks with Dulbecco's Modified Eagle's Medium and Ham's F-12 medium containing 1% penicillin and streptomycin (DMEM/F12; KeyGEN, Nanjing, China) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). When cell confluence reached to 80%, miR-340 mimic or miR-340 inhibitor (Sangon Biotechnology, Shanghai, China) was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. Next, lipopolysaccharide (LPS, 100 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) was employed to provoke KCs inflammation for 24 h.

Dual-Luciferase Assay

KCs transfected with Luciferase-labeled miR-640 mimic or miR-640 inhibitor (20 ng) *via* adeno-associated virus (AAV) loading were extracted following 72 h transfection. Then, Luciferase concentration in the transfected KCs was measured using Luciferase assay system (Promega, Madison, WI, USA) complied with manufacturer's protocol.

Mice

This investigation was approved by the Institutional Committee of People's Hospital of Zoucheng, and all experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the People's Hospital of Zoucheng. C57/B6J mice (aged six to eight-week-old, male, average weight: 21 g) were purchased from the Animal Center of People's Hospital of Zoucheng and were housed in cage. Animals were bred under the conditions of suitable temperature (22°C-26°C), humidity (50%-65%), and a 12 h light/dark cycle and provided with available normal food and water.

Acute Liver Injury and MiRNA Injection

The mice were randomly divided into four groups, namely, the control group (con, n=6), the ALI group (ALI, n=6), the mimic control group (mmc con, n=6), and the miR-640 mimic group (mmc, n=6). Briefly, miR-340 mimic control and miR-340 mimic were injected through the tai vein at 72 h before CCl_4 injection. Next, 25% CCl4 solution prepared with olive oil (200 μL) were used to treat mice to establish ALI model through intraperitoneal injection.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from macrophages with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) directed by the manufacturer's protocol. Reverse transcription was conducted to synthesize cDNA using PrimeScript™ RT Master Mix (Applied Biosystems, Foster City, CA, USA). qRT-PCR was conducted to quantify miR-340, Sigirr, P38, and 18s rRNA expression levels. Then, qRT-PCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). 18S rRNA was used for normalization. Finally, relative mRNA expression levels were quantified by the $2^{-\Delta\Delta Ct}$ methods. Primer sequences are listed in Table I.

Table I. Primer sequences.

Gene symbol	Sequence (5′-3′)
miR-340	F: GCGGTTATAAAGCAATGAGA R: GTGCGTGTCGTGGAGTCG
Sigirr	F: CAGTGTCCTGGTGCTCAACT R: GCTCGCCAAAGAGTGAAGGA
P38	F: CTGACCGACGACCACGTTC R: CTTCGTTCACAGCTAGGTTGC
GAPDH	F: TGACCTCAACTACATGGTCTACA R: CTTCCCATTCTCGGCCTTG
U6	F: CTCGCTTCGGCAGCACA F: AACGCTTCACGAATTTGCGT

Western Blot (WB) Analysis

Liver tissue and HLMs were harvested in lysis buffer. The proteins were isolated using a Total Protein Extraction Kit (Keygen, Nanjing, China), according to the manufacturer's instructions. Protein concentrations were measured with the enhanced bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China). After separation, transferring, and blocking with 5% skim milk for 1 hour at room temperature, the proteins were incubated overnight with anti-Sigirr (Abcam, Cambridge, MA, USA, 1:1000), anti-Bcl-2 (Abcam, Cambridge, MA, USA, 1:1000), anti-Bax (Abcam, Cambridge, MA, USA, 1:1000), anti-caspase-3 (Abcam, Cambridge, MA, USA, 1:1000), and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA, 1:2000). After washing with Tris-Buffered Saline and Tween-20 (TBST), the membrane was incubated with secondary antibody (YiFeiXue, Nanjing, China, 1:10000) for 1 h at room temperature. Finally, proteins were visualized and detected using an enhanced chemiluminescence (ECL) system.

Flow Cytometry Analysis

The apoptosis was determined using a 70-AP101-30 Annexin V-FITC/PI Apoptosis Kit (MultiSciences, Hangzhou, China) following the manufacturer's procedures. Liver cells were incubated with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) in the dark for 30 min. Apoptotic cells and viable cells were sorted using a fluorescence-activated cell sorting flow cytometer (BD FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

Histologic and Immumohistochemical (IHC) Staining

Liver tissues were fixated with 4% paraformaldehyde, and dehydration was gotten through different concentration alcohol. Then, tissues were embedded into paraffin and cut into sections (5 μm) using a rotary microtome. Hematoxylin-eosin staining and IHC were conducted using Hematoxylin and Eosin Staining Kit (Beyotime, Shanghai, China) and Rabbit Mouse HRP Kit (DAB, WE0316-JKS, Biolab, Berlin, Germany) following manufacturer's protocol, respectively. IHC was conducted for tissue sections with TNF-α, IL-1β, and IL-6. Then, images were visualized and gathered using a microscope.

Immunofluorescence (IF)

KCs (1×10^5/ well) underwent IF with Sigirr (Abcam, Cambridge, MA, USA, 1:300) overnight at 4°C, followed by Cy3-conjugated goat anti-rabbit IgG antibody (Abcam, Cambridge, MA, USA, 1:200) for 2 h at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI)-Fluoromount-G (Southern Biotechnology, Shanghai, China). Then, images were collected using a microscope.

Enzyme Linked Immunosorbent Assay (ELISA)

Serum were taken from mice. The cell medium and serum were centrifuged for 10 minutes, and then, supernatant was collected. ELISA detection was conducted using an ELISA Kit (MultiSciences, Hangzhou, China). Standard product was added in a 96-well plate with different concentration successively. Then, the colorant was seeded into each well and the samples were incubated in avoided light for 15 min. The termination solution was added to terminate the reaction and the absorbance (OD value) of each well was measured sequentially at 450 nm.

Statistical Analysis

Data were displayed as the means ± standard deviations. Comparison between the two groups were analyzed using Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Data were collected and assessed using GraphPad Prism (Version X; La Jolla, CA, USA). When $p<0.05$, differences were considered statistically significant.

Results

MiR-340 Alteration Specifically Regulates Sigirr Expression in ALI

Whether miR-340 changes in ALI following CCl_4 injection was firstly investigated. Then, liver tissue was reaped for RNA extraction to detect miR-340 level after ALI in mice within 7 days, finding that CCl_4-induced ALI triggered miR-340 decline at post injury, and the lowest expression level of miR-340 was at 3 days post injury (Figure 1A). Moreover, Sigirr RNA was also measured, and it was showed that Sigirr expression was decreased at post-ALI and the lowest Sigirr transcription level was at 3 days post-ALI (Figure 1B). To further verify whether miR-340 targets Sigirr to be involved in ALI pathogenesis, miR-340 mimic and miR-340 inhibitor were transfected into KCs, and transfec-

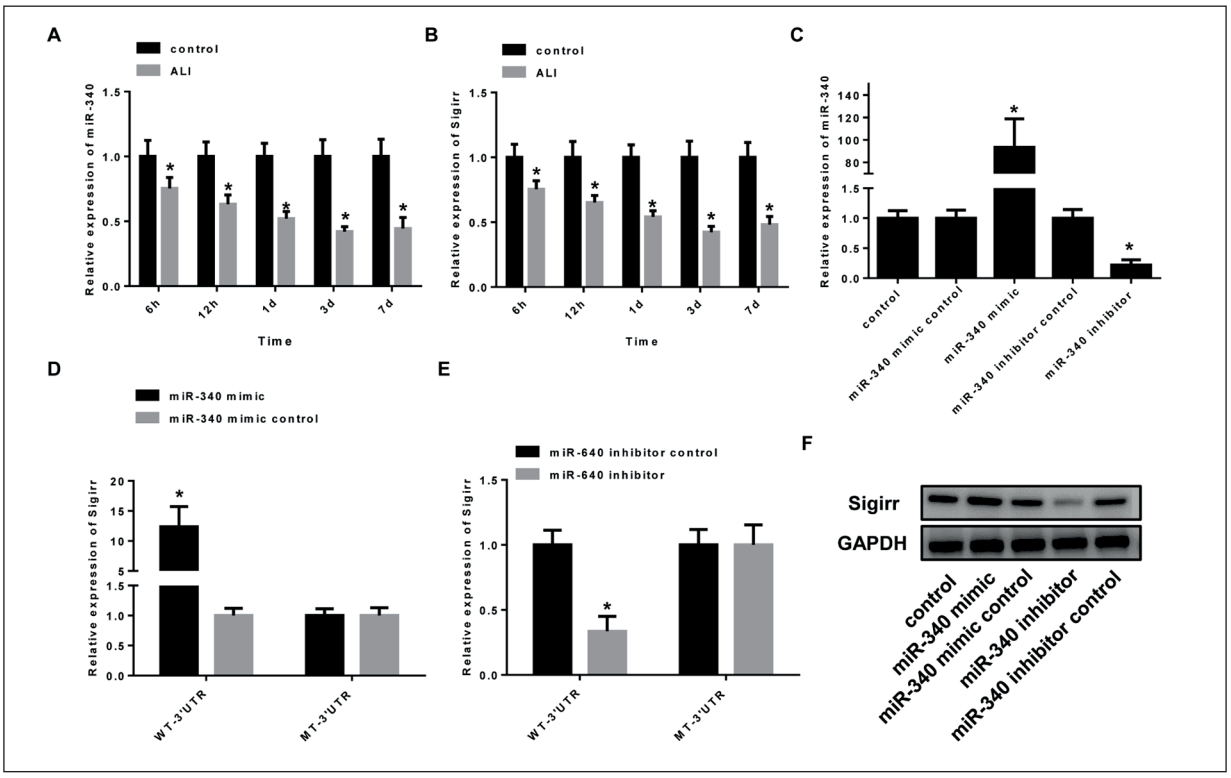

Figure 1. MiR-340 alteration specifically regulates Sigirr expression in ALI. **A,** Representative miR-340 RNA level at 6 h, 12 h, 1 day, 3 days, 7 days after ALI. **B,** Representative Sigirr RNA level at 6 h, 12 h, 1 day, 3 days, 7 days after ALI. **C,** Representative miR-340 RNA level transfected with miR-340 mimic control, miR-340 mimic, miR-340 inhibitor control, miR-340 inhibitor in KCs. **D,** Luciferase report of miR-340 mimic control and miR-340 mimic to WT-3'UTR and MT-3'UTR Sigirr RNA. **E,** Luciferase report of miR-340 inhibitor control and miR-340 inhibitor to WT-3'UTR and MT-3'UTR Sigirr RNA. **F,** Representative Sigirr protein level transfected with miR-340 mimic control, miR-340 mimic, miR-340 inhibitor control, miR-340 inhibitor in KCs. "*" means *vs.* control group with statistical significance.

tion level was detected using Dual-Luciferase reporter. The results exhibited that miR-340 mimic increased miR-340 level and miR-340 inhibitor repressed miR-340 level (Figure 1C). Then, miR-340 mimic upregulated Sigirr RNA level and miR-340 inhibitor decreased Sigirr expression (Figure 1D, 1E). Besides, Sigirr protein was reduced *via* miR-340 inhibitor but Sigirr expression was elevated in the miR-340 mimic group (Figure 1F). Therefore, the above results demonstrate that miR-340 interacts with Sigirr transcription in ALI.

MiR-340 Participates KCs Inflammation Response Via Modulating Sigirr

Next, whether miR-340 overexpression or inhibition influences KCs inflammation was explored *via* targeting Sigirr. The expression of Sigirr was visualized by immunofluorescence staining, and it was found that the expression of Sigirr following mimic treatment was significantly enhanced, while the expression of Sigirr was decreased *via* inhibitor administration KCs activation or not (Figure 2A). To examine the inflammation level of KCs activation, inflammatory factors including TNF-α, interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and monocyte chemo-attractant protein-1(MCP-1) in cell free supernatant were measured *via* ELISA. It was displayed that miR-340 overexpression slightly reduced the expressions of TNF-α, IL-1β, IL-6, and MCP-1, and miR-340 inhibition enhanced the levels of TNF-α, IL-1β, IL-6, and MCP-1. After KC activation, miR-340 mimic remarkably restrained the release of TNF-α, IL-1β, IL-6, and MCP-1, while miR-340 inhibitor significantly aggravated the levels of the inflammation factors (Figure 2B-2E). Moreover, P38 expression in RNA level was measured, showing that increased miR-340 evidently declined P38 level but inhibition of miR-340 effectively upregulated P38 transcription after LPS treatment or

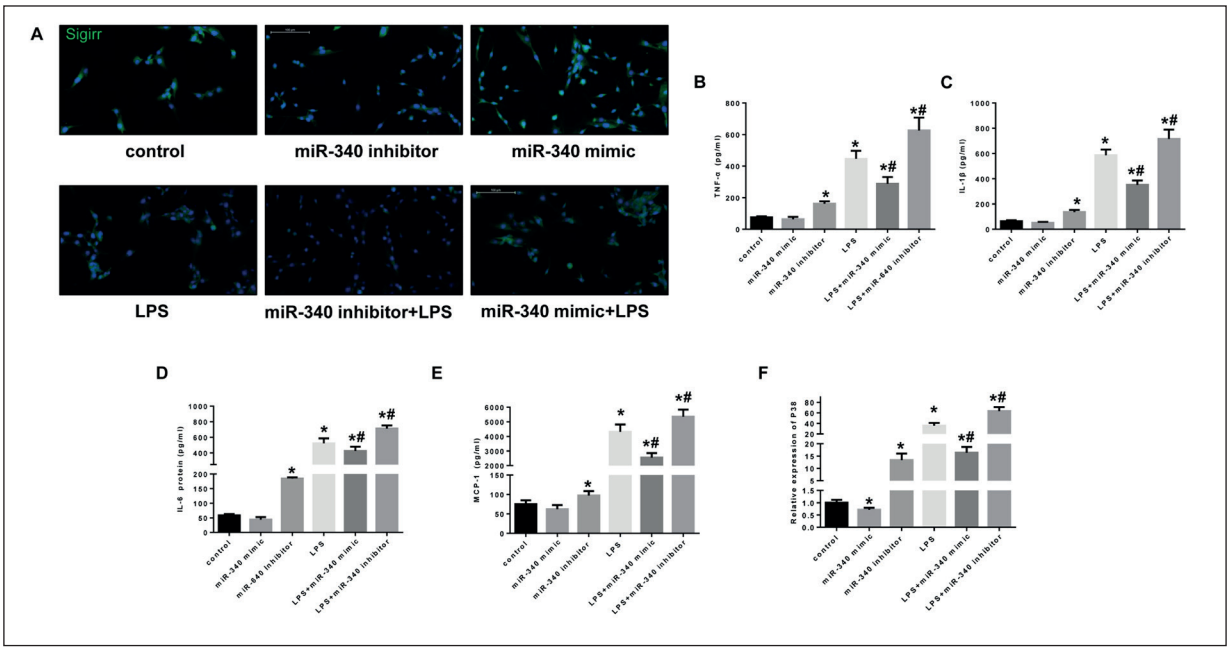

Figure 2. MiR-340 participates in KC inflammation response *via* modulating Sigirr. **A,** Representative immunofluorescence of Sigirr in control, miR-340 mimic, miR-340 inhibitor, LPS, LPS+miR-340 mimic and LPS+miR-340 inhibitor group (magnification: 200×). **B-E,** Representative ELISA of TNF-α, IL-1β, IL-6 and MCP-1 in control, miR-340 mimic, miR-340 inhibitor, LPS, LPS+ miR-340 mimic and LPS+miR-340 inhibitor group. **F,** Representative RNA level of P38 in the control, miR-340 mimic, miR-340 inhibitor, LPS, LPS+miR-340 mimic and LPS+miR-340 inhibitor group. "*" means *vs.* control group with statistical significance. "#" means *vs.* LPS group with statistical significance.

not (Figure 2F). Hence, the results showed that miR-340 participated in KC inflammation by regulating Sigirr and P38 pathway.

Increased miR-340 Alleviates Inflammation and Apoptosis in ALI Mice

Next, the ameliorative effect of miR-340 increase on ALI was evaluated. Following transfection of miR-340 mimic *via* tail vein injection, the mice were used for ALI model establishment. Then, immunoblotting exhibited that the protein level of Sigirr was increased following miR-340 mimic transfection, while the protein level of Sigirr was decreased significantly after ALI, and miR-340 increase rescued the decline of Sigirr in ALI (Figure 3A). Immunohistochemistry displayed that TNF-α, IL-1β, and IL-6 did not change significantly after miR-340 overexpression, but the expressions of TNF-α, IL-1β, and IL-6 were increased significantly after ALI, while increased miR-340 promoted a broad restriction effect on the levels of the multiple inflammatory mediators (Figure 3B). Besides, to explore that miR-340 restraining inflammation potentially affects the apoptosis of liver tissue, apoptotic cells detection was conducted using flow cytometry assay, exhibiting that apoptosis level in liver tissue increased significantly after ALI, while miR-340 mimic treatment significantly reduced apoptosis level (Figure 3C). Furthermore, apoptosis-related factors were detected by Western blotting, showing that the expressions of caspase 3 and Bax were significantly increased but Bcl-2 declined in the ALI group. However, after miR-340 overexpression, the expression levels of caspase 3 and Bax were significantly suppressed and Bcl-2 was elevated, which resulted in the decreased apoptosis level (Figure 3D). Therefore, the above results show that miR-340 mimic decreases inflammation and apoptosis of liver tissues after ALI.

MiR-340 Overexpression Protects Liver Function and Histological Structure

Finally, the hepatic histology and function were examined with treatment of miR-340 mimic following ALI. HE staining visualized that there was intact cell morphology, prominent nucleus, and no inflammation or necrosis hepatic tissue in control and miR-340 mimic groups. However, CCl_4 provoked diffuse lesion and necrosis of hepatic cells, resulting in hemorrhage, swelling, and inflammatory cell infiltration. Through the administration of miR-640 mim-

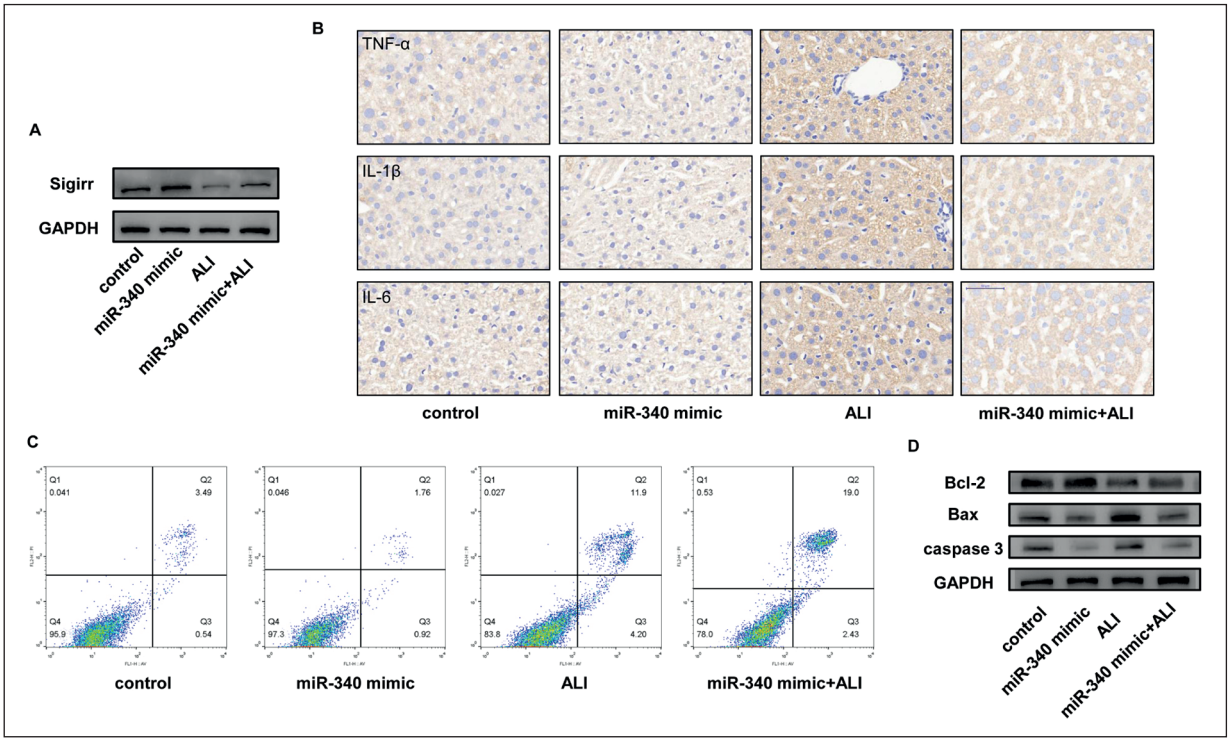

Figure 3. Increased miR-340 alleviates inflammation and apoptosis in ALI mice. **A,** Representative western blotting of Sigirr in the control, miR-340 mimic, ALI and ALI+miR-340 mimic group. **B,** Representative IHC of TNF-α, IL-1β and IL-6 in the control, miR-340 mimic, ALI and ALI+miR-340 mimic group (magnification: 200×). **C,** Representative flow cytometry assay of apoptosis in the control, miR-340 mimic, ALI and ALI+miR-340 mimic group. **D,** Representative Western blotting of Bcl-2, Bax and caspase 3 in the control, miR-340 mimic, ALI and ALI+miR-340 mimic group. "*" means *vs.* control group with statistical significance. "#" means *vs.* ALI group with statistical significance.

ic, CCl_4-mediated injury in hepatic pathomorphology was significantly corrected (Figure 4A). Moreover, hepatic function was examined through serum transaminase and bilirubin. The results showed that CCl_4 induction increased serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) concentration, and total bilirubin (TBIL) level, but enhancing miR-340 treatment reduced serum ALT, AST concentration, and TBIL level resulted from CCl_4 instigation (Figure 4B, 4C). Therefore, increased miR-340 expression ameliorates hepatic histology and function following ALI.

Discussion

Inflammatory response is a complex defense process caused by various factors, such as chemical stimulation, physical injury, and bacterial infection[19]. Normal inflammation protects the body, while excessive inflammation can lead to cell and tissue damage. Inflammation mediated-ALI is caused by the large release of pro-inflammatory cytokines due to external stimulation[20]. MiRNAs target a broad range of genes and regulate even more than 30% of human genes, which are involved in the regulation of various cellular processes, such as cell cycle, apoptosis, hematopoietic stem cell differentiation, metabolism, and tumor metastasis[21]. Several miRNAs have been proved to be possible biomarkers of ALI, and the data of this study showed a significant decrease in the expression of miR-340 after ALI, proving that miR-340 can also be used as a biomarker and therapeutic target for ALI. MiR-340 can serve as an inhibitor of a variety of tumors, which is closely related to the occurrence and metastasis of tumors, the invasion ability of carcinoma cells, and cytoskeletal reconstruction[22,23]. The expression of miR-340 is inhibited in malignant tissues compared to normal/benign tissues, and the reduction of miR-340 level can lead to tumor development and metastasis.

In this study, it was hypothesized that miR-340 in ALI may also play a role in preventing injury effects during inflammation by activation

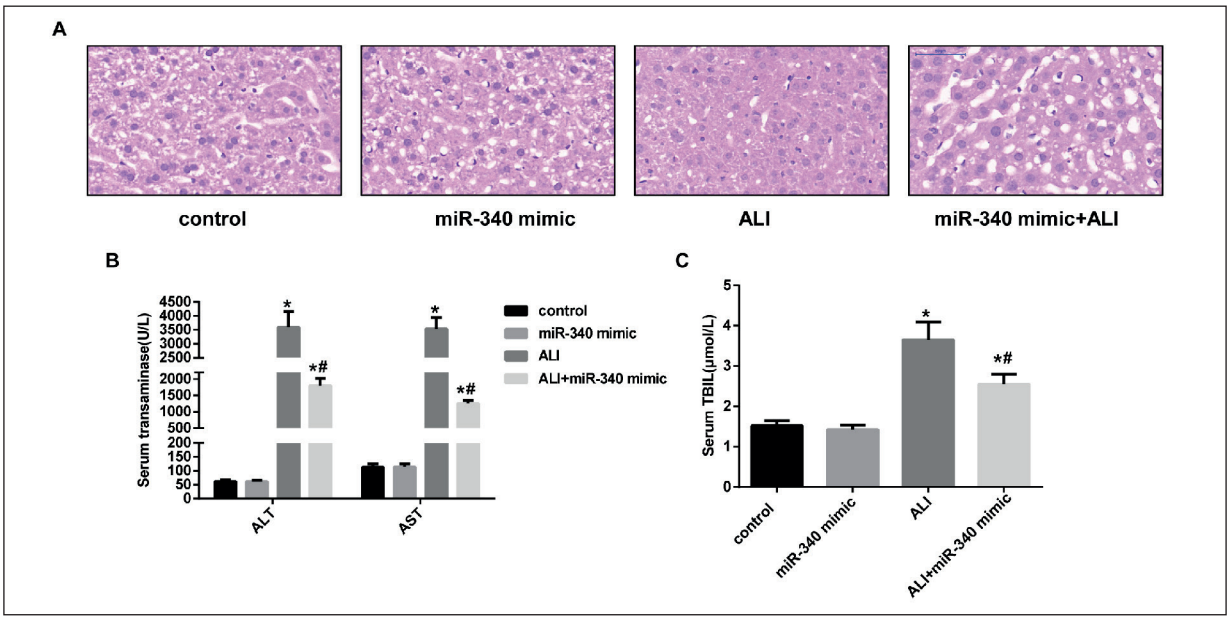

Figure 4. MiR-340 overexpression protects liver function and histological structure. **A,** Representative HE staining in the control, miR-340 mimic, ALI and ALI+miR-340 mimic group (magnification: 200×). **B,** Representative serum ALT and AST level in the control, miR-340 mimic, ALI and ALI+miR-340 mimic group. **C,** Representative serum TBIL detection in the control, miR-340 mimic, ALI and ALI+miR-340 mimic group. "*" means *vs.* control group with statistical significance. "#" means *vs.* ALI group with statistical significance.

of Sigirr RNA and exerting anti-inflammation effect. Therefore, miR-340 and Sigirr levels were measured in ALI *via* CCl_4 stimulation within 7 days, and it was found that the expression of miR-340 was significantly decreased. Consistently, Sigirr level was decreased. The overexpressed or inhibitory effect of miR-340 in KCs was further detected, and it was found that miR-340 overexpression upregulated the expression of LRP1 and inhibited inflammation. Multiple studies have proved that Sigirr and inhibiting P38 pathway are involved in the mediation of anti-inflammatory response. Besides, it was proved that inhibiting expression of P38 after inflammatory response and Sigirr upregulation in KC inflammation was realized *via* miR-340 overexpression. Hence, the concentrations of pro-inflammatory factors and chemokines, such as TNF-α, IL-1β, IL-6, and MCP-1 released from miR-340 mimic treated KCs were significantly decreased. Besides, CCl_4-induced ALI disease model was established in mice, and miR-340 mimic treatment was applied to evaluate the mend of ALI. *In vivo*, we detected that miR-340 increase promoting Sigirr expression downregulated P38 pathway activation and played an anti-inflammatory role. Importantly, it was found that miR-340 mimic blocked the inflammation-mediated elevation of TNF-α, IL-1β, and IL-6 in liver tissue, suggesting that miR-340 overexpression also reduced the infiltration of peripheral inflammatory cells into liver tissue. Moreover, CCl_4 stimulates KCs to secrete large amounts of TNF-α, which directly mediates apoptosis by binding to TNF-α receptor 1, leading to Bax oligomerization and insertion into mitochondria, initiating apoptosis of the mitochondrial pathway. Therefore, a group number of apoptotic cells can be detected in ALI tissue. However, miR-340 increase could effectively decrease the level of TNF-α and downregulate the expression of pro-apoptosis factors. Therefore, it was found that the apoptosis level in liver tissues was significantly mended following miR-340 mimic treatment in ALI. MiR-340 increase ultimately improved liver function and protected liver tissue structure. Therefore, increased miR-340 finally lowered the serum content of ALT, AST, and TBIL after ALI and reduced the area of tissue damage caused by inflammation in liver tissue, thus improving liver function and protecting liver tissue structure.

Conclusions

To sum up, miR-340 increase mediates liver protection in CCl_4-induced ALI, miR-340 overexpression promotes Sigirr and regulates P38 pathway to play an antagonism of inflammatory infiltration in the liver. Moreover, miR-340 mitigates the levels cell apoptosis in liver tissue, so as to improve hepatic function and prevent excessive damage to hepatic structure.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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